

New and Common Haplotypes Shape Genetic Diversity in Asian Tiger Mosquito Populations from Costa Rica and Panamá

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J. Econ. Entomol. 108(2): 761–768 (2015); DOI: 10.1093/jee/tou028

ABSTRACT The Asian tiger mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae), is a vector of several human pathogens. *Ae. albopictus* is also an invasive species that, over recent years, has expanded its range out of its native Asia. *Ae. albopictus* was suspected to be present in Central America since the 1990s, and its presence was confirmed by most Central American nations by 2010. Recently, this species has been regularly found, yet in low numbers, in limited areas of Panamá and Costa Rica (CR). Here, we report that short sequences (~558 bp) of the mitochondrial cytochrome oxidase subunit I (COI) and NADH dehydrogenase subunit 5 genes of *Ae. albopictus*, had no haplotype diversity. Instead, there was a common haplotype for each gene in both CR and Panamá. In contrast, a long COI sequence (~1,390 bp) revealed that haplotype diversity (\pm SD) was relatively high in CR (0.72 ± 0.04) when compared with Panamá (0.33 ± 0.13), below the global estimate for reported samples (0.89 ± 0.01). The long COI sequence allowed us to identify seven (five new) haplotypes in CR and two (one new) in Panamá. A haplotype network for the long COI gene sequence showed that samples from CR and Panamá belong to a single large group. The long COI gene sequences suggest that haplotypes in Panamá and CR, although similar to each other, had a significant geographic differentiation ($K_{st} = 1.33$; $P < 0.001$). Thus, most of our results suggest a recent range expansion in CR and Panamá.

KEY WORDS mitochondrial COI, ND5, *Aedes albopictus*, invasive species, dengue vectors

Introduction

The Asian tiger mosquito, *Aedes albopictus* (Skuse) (Diptera: Culicidae), is an invasive insect species that has been expanding globally in the past 150 yr (Lounibos 2002, Benedict et al. 2007, Bonizzoni et al. 2013). Its successful expansion is mainly because of its desiccation-tolerant eggs and adaptation to small aquatic habitats (Lambrechts et al. 2010). These characteristics allowed *Ae. albopictus* to inhabit artificial water containers that promote its close interaction with humans (Bonizzoni et al. 2013). Moreover, *Ae. albopictus* aggressive biting behavior (Ponlawat and Harrington 2005) and vectorial competence, allow its females to transmit a wide array of arboviruses (Benedict et al. 2007, Paupy et al. 2009), most notoriously dengue virus (Lambrechts et al. 2010) and Chikungunya

virus (Paupy et al. 2009). Moreover, for dengue virus, mosquitoes can get infected vertically, i.e., without involving vertebrate hosts (Martins et al. 2012).

Several studies have suggested the geographical origin of *Ae. albopictus* to be in Southeast Asia (Hawley et al. 1987, Khambhampati et al. 1991, Rai 1991, Porretta et al. 2012) from where it likely invaded, mainly by means of maritime trade, most of East Asia before the end of the 19th century (Lounibos 2002). Nevertheless, *Ae. albopictus* gained notoriety in the 1980s, after becoming established in Harris County, TX, where it became a dominant vector species in the Houston area (Sprenger and Wuithiranyagool 1986). Subsequent molecular genetics studies, and additional ecological evidence, suggested Japan as the likely place for the origin of this infestation (Hawley et al. 1987, Khambhampati et al. 1991, Rai 1991, Lounibos 2002, Bonizzoni et al. 2013). The detection of the Asian tiger mosquito in United States was not a mere description of a range expansion, it highlighted how the expansion and establishment of this species, like many other invasive species, has been driven by the intensification of global commodity trade (Bonizzoni et al. 2013), first by its detection at seaports (Eads 1972) and its subsequent detection and establishment at the final destination of trade commodities (Reiter and Darsie 1984, Sprenger and Wuithiranyagool 1986). For example, *Ae. albopictus* was already present in Albania in the late 1970s, a time when Albania was the main European commercial partner of China, a country within the native range of *Ae. albopictus* (Adhami

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and Reiter 1998). Similarly, in the mid-1980s, the species was detected in São Paulo, Brazil (Forattini 1986), the economic heart of South America. Currently, *Ae. albopictus* has spread over Europe, Oceania, and reports of its presence and establishment all over Africa are becoming increasingly common, with extensive documentation of trade playing a major role on *Ae. albopictus* expansion (Bonizzoni et al. 2013).

In the New World, *Ae. albopictus* spread to Mexico by the early 1990s (Rai 1991, Lounibos 2002) was suspected in most Central American countries by the late 1990s (Eritja et al. 2005), with all countries confirming its presence by 2010 (Bonizzoni et al. 2013).

In Panamá, *Ae. albopictus* was first detected in 2002, in the “24 de diciembre” neighborhood of Panamá city (ICGES 2003). According to dengue entomological surveys from Panamá’s Ministry of Health, *Ae. albopictus* has been mainly found in urban settings (Espino et al. 2011, Díaz 2012). Nevertheless, from 2002, *Ae. albopictus* has been monotonically increasing its abundance, having a house index close to 0.5 % in 2013 (Díaz 2012). In Costa Rica (CR), *Ae. albopictus* larvae were first recorded during 2007 in coconut shells at Siquirres, in the Atlantic basin of CR (Marín et al. 2009). Incipient *Ae. albopictus* populations, i.e., persistent but low densities per trapping effort, have only been observed in rural settings in the Atlantic basin of CR (Marín et al. 2009, Calderón Arguedas et al. 2012, Marín Rodríguez et al. 2013). *Ae. albopictus* has not been detected in the Central Valley, and is rare across urban and rural settings in the Pacific basin of CR (Morice Trejos et al. 2010).

The ecology of *Ae. albopictus* in CR and Panamá suggests that populations in these two nations, although not established in the sense of widespread infestations like the ones observed in Harris County, TX, in the mid-1980s (Sprenger and Wuithiranyagool 1986) and everywhere else the tiger mosquito is now established (Bonizzoni et al. 2013), are incipient, given their persistence, yet in low abundance, at specific locations. It is unclear whether this reflects the relative recent invasion of these territories by *Ae. albopictus*. This hypothesis can be tested with tools from molecular genetics, where a recent invasion would be more likely associated with low genetic diversity (Avisé 1994). From a broader ecological perspective, it is expected that Panamanian *Ae. albopictus* populations potentially have a greater genetic diversity given the fundamental role the Panamá Canal plays in global commerce, with ships containing goods from all over the world regularly crossing it (Llacer 2005), and needing to spend at least 3 d within the Panamanian territory. This situation poses a potentially higher propagule pressure (Lounibos 2002) in Panamá than CR, i.e., the recruitment of new individuals from abroad that can contribute unique genetic material is more likely to occur in Panamá, thus leading to the expectation of more introgressions, and perhaps haplotype diversity, in Panamá than CR, considering that both countries have similar strategies for dengue mosquito control.

Mitochondrial DNA genes are ideal genetic markers to test hypothesis about ancestry and demographic

changes in populations (Avisé 1994) because of their lack of recombination, uniparental inheritance, high mutation and nucleotide substitution rates, and the well-defined effective population size of one-fourth nuclear genes (Avisé 1994, Birungi and Munstermann 2002, Usmani-Brown et al. 2009). Moreover, mitochondrial genes have been frequently used in studies seeking inferences about the genetic relationships of *Ae. albopictus* (Birungi and Munstermann 2002, Mousson et al. 2005, Usmani-Brown et al. 2009, Delatte et al. 2011, Kamgang et al. 2011, Porretta et al. 2012, Raharimalala et al. 2012, Navarro et al. 2013, Zhong et al. 2013), therefore making easy the comparison with samples from several places in the globe. Here, we thus report: 1) haplotypes for the NADH dehydrogenase subunit 5 (ND5) and cytochrome oxidase subunit 1 (COI) mitochondrial genes present in incipient *Ae. albopictus* populations of CR and Panamá, 2) analyze mitochondrial COI and ND5 sequences of samples from CR and Panamá, to explore genetic differences between mosquitoes from these neighboring countries and propose a possible geographical origin of *Ae. albopictus* populations in CR and Panamá, nations outside the original native range of this invasive insect. In our analysis, we also considered two nonindependent sequences of the COI gene, a short (558 bp) and a long (1,390 bp) sequence, where the short sequence is embedded within the longer sequence, to increase the precision of genetic structure estimates in *Ae. albopictus* from Panamá and CR.

Materials and Methods

Mosquito Sampling. Mosquitoes were collected in CR by Departamento de Control de Vectores, Ministerio de Salud, CR, at an organic pineapple farm “Finca Corsicana” located in La Virgen de Sarapiquí, Sarapiquí County, Province of Heredia, CR (10° 26′03.80″ N, 84° 07′14.75″ W). This farm has had a persistent infestation by *Ae. albopictus*, probably associated with the pesticide-free nature of its agricultural production. For the collection, three CDC backpack aspirators (Clark et al. 1994) were operated by personnel of CR’s Ministry of Health. After 6 h of operation, we collected 58 adult females on a surface of 1.6 ha of land cultivated with pineapples, surrounded by patches of tropical rainforest, the native vegetation of the area. The sampling was performed in December 2012.

In Panamá mosquitoes were collected in urban areas from July to September 2012 by personnel from the Departamento de Control de Vectores, Ministerio de Salud, República de Panamá. Mosquitoes were collected in Chepo (9° 9′52″ N, 79° 5′43.37″ W), Province of Panamá and Arco Iris (9° 20′21.39″ N, 79° 53′26.80″ W), Province of Colón, all locations with persistent infestations by *Ae. albopictus* (Espino et al. 2011). Assuming a house index of 0.5%, 10,057 houses were surveyed, expecting to find around 50 houses with *Ae. albopictus*.

In Panamá, the sampling procedure was performed following the Ministry of Health protocol for dengue entomological surveillance. Briefly, trained crews

visited randomly selected households in each location and collected all containers with larval mosquitoes. These containers were then processed by the ICGES Department of Medical Entomology, where all larvae from a positive container were transferred into a 1-liter container with 1 g of yeast as a food source. Pupae from the surveys and containers were then transferred to emergence containers kept at 25°C and with an 80–90% relative humidity. For the molecular analysis, we considered a single individual per positive house, totaling 3 from Chepo and 15 from Arco Iris.

In both Panamá and CR, adult mosquitoes were killed by flash-freezing the individuals at –5°C, before an identification based on morphological characters (Rueda 2004). Morphological characters included, the narrow white medial longitudinal stripe on the scutum, but also other major morphological characters; the V-shaped patch of white scales on the mesepimeron, the lack of white scales on the clypeus, the white transverse bands on the anterior abdominal terga, and the complete white rings in the last tarsal segment of tarsus III. For preservation, mosquitoes were kept in ethanol at 99% shortly after the identification process.

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification and Sequencing. We analyzed 58 adult females from CR and 18 adults from Panamá (2 females and 1 male from Chepo, and 15 females from Arco Iris) for molecular analysis. Three legs from each adult were placed in a 1.5-ml PCR reaction tube. Each sample was homogenized in a mixture of extraction solution (20 µl) + tissue preparation solution (5 µl; REDEExtract-N-Amp™ Tissue PCR Kit; SIGMA, St. Louis, MO) for DNA extraction. The solution was heated at 95°C for 3 min and neutralized (Kawada et al. 2011).

PCR amplification targeted two mtDNA gene fragments: a 406-bp fragment of ND5 and a 1,390 bp fragment of COI excluding primer sequences. One primer set for ND5 and two primer sets for COI were used: for ND5, ND5albof (5'-TCCTTAGAATAAAATCCC GC-3') and ND5albor (5'-GTTTCTGCTTTAGTT-CATTCTTC-3'; Birungi and Munstermann 2002); for the upstream COI, albo1454F (5' GGTCAACAAAT-CATAAGATATTGG 3') and albo2160R (5' TAAACTT CTGGATGACCAAAAAATCA 3'); for the downstream COI, albo2027F (5' CCCGTATTAGCCGGAGCTAT 3') and albo2886R (5' ATGGGGAAGAAGGAGTT CG 3'; Zhong et al. 2013). Each 10 µl of the master mix contained 1 × PCR buffer, 0.2 mM dNTP, 0.2 µM of each primer, and 0.25 unit of TaKaRaExTaq, and 1 µl of template DNA. The temperature profile for ND5 consisted of an initial denaturation at 98°C for 5 min, followed by 35 cycles at 95°C for 1 min, 60°C for 1 min, 72°C for 1 min 30 s, and then a final extension at 72°C for 3 min. The profile for both primer sets of COI consisted of a 94°C for 3 min as initial denaturation, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 1 min, then final extension at 72°C for 6 min (Zhong et al. 2013). To confirm amplification, 4 µl of the PCR products were mixed with 2 µl of EZ-Vision (Amresco Inc., Solon, OH) and loaded for electrophoresis with 2% agarose gel. The bands were

visualized with an UV transilluminator. When the amplification was confirmed, remaining PCR products (~5 µl) were treated with 0.2 µl of ExoSAP-IT (Affymetrix Inc., Sunnyvale, CA) for 30 min at 37°C followed by 15 min at 80°C in a thermal cycler.

The purified products were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA). Each reaction mix contained 0.5 µl of Big Dye terminator, 1.8 µl of 5× sequencing buffer, 0.2 µM of forward or reverse primer used at PCR amplification, and 1 µl of purified PCR products. The reaction consisted of an initial denaturation step for 1 min at 96°C, followed by 25 cycles of 10 s at 96°C, 5 s at 50°C, and 4 min at 60°C. Sequencing reaction products were purified by ethanol precipitation method and dissolved in 10 µl of Hi-Di Formamide (Applied Biosystems). The product was denatured at 95°C for 2 min and rapidly cooled on ice, and upstream and downstream sequences were analyzed on an ABI 3730 or ABI 3130 automatic sequencer (Applied Biosystems). We separated the 1,390 bp into upstream and downstream sequences, and amplified them separately. Because the amplified regions overlapped, we connected the two streams to build the sequence for each sample.

Data Analysis. *Processing of ND5 and COI Sequences.* ND5 and long COI gene sequences obtained from our mosquito samples were manually aligned using MEGA 5.2.1 (Tamura et al. 2011). Newly obtained long COI sequences were trimmed at both ends and rearranged into short sequences (short COI, 558 bp), consistent with those presented by Mousson et al. (2005) excluding the primer nucleotides. Haplotypes from long COI (1,390 bp) sequences presented by Zhong et al. (2013) were considered into subsequent analyses and also trimmed into short COI sequences for haplotype diversity comparison. Published ND5 and short COI haplotype sequences were obtained from GenBank and ND5 sequences not present in GenBank were extracted from a report by Navarro et al. (2013). COI sequences >500 bp were selected for the short COI analysis. Each haplotype was identified by calculating the number of different sites between each sequence pair using MEGA 5.2. This allowed us to identify sequences sharing the same exact haplotype. Haplotype codes for ND5, short, and long COI sequences are presented in the online only Supp Tables 1–3 (online only), respectively.

Haplotype Networks. Haplotype networks were built with the statistical parsimony algorithm implemented in TCS (Clement et al. 2000). Haplotype networks show haplotype frequencies in each population and their relatedness, which is useful information to infer the plausible geographical origin of a population. It is expected that ancestral populations should have a larger allele diversity than colonizing populations, which are expected to exhibit the loss of rare haplotypes or to present new haplotypes linked to the likely ancestral haplotypes (Clement et al. 2000). For the analyses, we considered the frequency of haplotype reported in previously published studies used for comparison. Gaps were treated as missing data, and the parsimony threshold probability was set at 0.95%.

Population Genetic Analyses with Long COI Gene Sequences. The long COI sequences of CR, Panamá, and the 12 populations studied by Zhong et al. (2013) were used to estimate several parameters useful to describe the genetic structure of *Ae. albopictus*.

A first group of parameters assessed molecular diversity. These included the number of polymorphic sites, haplotype diversity, and nucleotide diversity.

A second group of parameters assessed the genetic structure of the studied samples. We studied pairwise geographical subdivision in our samples with the K_{st} statistic, a statistic able to detect geographic differentiation with just 10 samples per locality (Hudson et al. 1992). Significance of the K_{st} was tested through Markov Chain Monte Carlo (1,000 replications). Neutrality tests were conducted via the estimation of Tajima's D, a test for population expansion (Tajima 1989). Briefly, Tajima's D has a null hypothesis of neutral variation when it is not different from 0, and alternative hypotheses of 1) a recent population bottleneck (or contraction) when it is significantly positive, or 2) a recent population expansion when it is significantly negative (Tajima 1989).

All population genetic parameters mentioned in this section were estimated with the software DnaSP5.10 (Librado and Rozas 2009).

Results

Haplotype Diversity. We were able to successfully sequence 57 samples from CR and 16 samples from Panamá for the ND5 and COI gene sequences. For ND5, all our 73 samples had a unique haplotype NH3 (Supp Table 1 [online only]). This haplotype had the same sequence of haplotype 3 from a previous study (Birungi and Munstermann 2002), which is globally widespread (Supp Table 1 [online only]).

All samples from CR and Panamá had a unique short COI haplotype, SH03 (Supp Table 2 [online only]). This haplotype had the same sequence of H3 in Mousson et al. (2005), which is globally widespread. In contrast, the long COI sequence (1,390 bp) revealed seven haplotypes in CR. Five of them (H67–H71) were new haplotypes, and the remaining two, H17 and H37, had already been described (Supp Table 3 [online only]). In Panamá, H37 and a new haplotype, H72, were observed, thus totaling two haplotypes (Supp Table 3 [online only]). Accession codes for the new haplotypes are presented in Table 1. Haplotype and nucleotide diversity for the long COI gene were larger in CR than Panamá, and when compared with the diversity observed in other areas, they were low in Panamá, but relatively high in CR (Table 2).

In total, 18 haplotypes were identified when combining data on short COI haplotypes from Zhong et al. (2013) and our samples from CR and Panamá (SH1–SH18; Supp Table 2 [online only]). In contrast, the long COI sequences for the same data had a total of 72 haplotypes (Supp Table 3 [online only]). The number of haplotypes from the long COI sequence was linearly

Table 1. Codes and accession numbers for the long COI gene (1,390 bp) haplotypes found in Costa Rica (CR) and Panamá (PN)

Haplotype	GenBank access no.	CR	PN
H17	KC690912	3	0
H37	KC690932	14	13
H67	AB907796	24	0
H68	AB907797	13	0
H69	AB907798	1	0
H70	AB907799	1	0
H71	AB907800	1	0
H72	AB907801	0	3

Table 2. Haplotype (Hd) and nucleotide (π) diversity in long sequences of *Ae. albopictus* COI gene

Site code	Site name	N	Variable sites	No. of haplotypes	Hd (SD)	$\pi \times 10^{-4}$
GZ	Guanzhou, China	32	6	6	0.59 (0.09)	5
XM	Fujian, China	29	11	11	0.82 (0.05)	16
JS	Jiangsu, China	30	3	2	0.37 (0.08)	8
TW	Taiwan	30	8	8	0.59 (0.10)	6
JP	Japan	15	3	3	0.59 (0.08)	8
SG	Singapore	36	11	11	0.74 (0.07)	28
IT	Italy	32	7	11	0.81 (0.06)	14
LA01	Los Angeles 01	15	9	6	0.83 (0.06)	21
LA11	Los Angeles 11	34	8	6	0.51 (0.09)	13
NJ	New Jersey	30	4	5	0.54 (0.10)	7
TX	Texas	31	12	9	0.72 (0.08)	14
HW	Hawaii	32	8	8	0.70 (0.07)	10
CR	Costa Rica	57	4	7	0.72 (0.04)	10
PN	Panamá	16	3	2	0.33 (0.13)	7
–	All Areas	419	36	72	0.89 (0.01)	19

correlated with the number of haplotypes from the short COI sequence, the slope of a linear regression, b , not being different from 1 but with an intercept, a , different from 0 when there is one haplotype (Fig. 1). This result indicates that, as expected, the number of haplotypes increased linearly with sequence length and proportionally with the number of short sequence haplotypes. Figure 1 also highlights that for CR, given the large number of mosquito samples, the number of haplotypes was unusually large for the long COI sequence, reflecting that haplotype number has an error expected to be proportional to sample size. The linear regression was able to explain 47% of the variability in the relationship between the number of long and short COI haplotypes (Fig. 1).

Haplotype Networks. The long COI network (Fig. 2) showed the five newly identified haplotypes from CR were placed near each other and where connected with H17 and H37, which are relatively widespread haplotypes (see Supp Table 3 [online only]). H37 was a haplotype shared with Panamá. A new haplotype found in Panamá, H72, was linked with the most common haplotype, H03 but not with H37. We did not generate haplotype networks for ND5 and the short COI gene sequence given their lack of diversity.

Population Genetic Structure. The pairwise K_{st} tests (Table 3) showed that all population pairs were significantly differentiated ($K_s = 1.673$; $K_{st} = 0.348$;

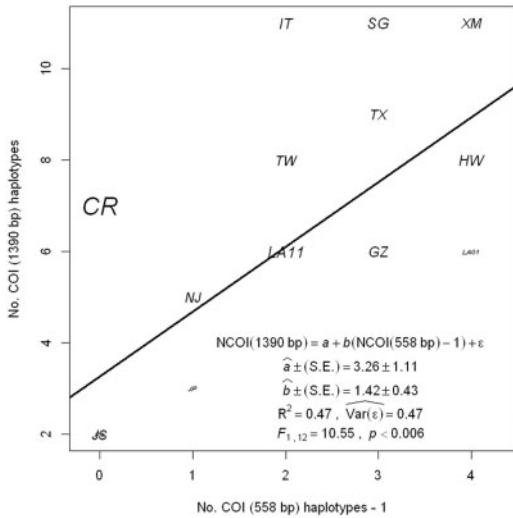


Fig. 1. Haplotype number for the long COI gene as function of the short COI gene by sampled location. For site code, see Table 2. In the plot, character size is proportional to the number of sampled mosquitoes, and the solid line indicates the estimated linear regression. The regression equation is presented inside the plot, where the number of long sequence COI haplotypes (NCOI [1,390 bp]) is a function of the number of short sequence COI haplotypes (NCOI [558 bp]) minus one. The one is subtracted from NCOI (558 bp) to interpret the intercept as the NCOI (1,390 bp) when there is one haplotype in NCOI (558 bp). Parameter estimates for the intercept, a , the slope, b , and the error variance $\text{VAR}(\epsilon)$, are also presented in the figure. The error was assumed to be normally distributed. In the linear regression, weights proportional to the inverse of the samples used to estimate the number of haplotypes were used, following the assumption that haplotype sampling has an error proportional to sample size.

$P < 0.001$). Tajima's D based on the long COI sequences suggests that populations in both CR ($D = 1.43$; $P > 0.05$) and Panamá ($D = 0.23$; $P > 0.005$) were in genetic equilibrium and neither expanding or contracting, as expected under the neutral mutation hypothesis once a population is established (Tajima 1989)

Discussion

Our phylogeographic analysis revealed some interesting patterns about *Ae. albopictus* in Panamá and CR. The first conclusion is that mosquitoes belong to a large group, which, based on inferences from ND5 and short COI sequences, represent the most common and widespread haplotypes reported for each of those two gene sequences (Birungi and Munstermann 2002, Mousson et al. 2005, Usmani-Brown et al. 2009, Raharimalala et al. 2012). The increased accuracy in the inferences brought by the use of long COI sequences, on the one hand, supports that *Ae. albopictus* in CR and Panamá belongs to a large group of haplotypes. Haplotypes in Panamá and CR were closely related with each other, one of the eight haplotypes found in our samples being

common in the two countries, H37, and also in the two Panamanian sampling locations. The five newly identified haplotypes from CR were placed near each other, and these haplotypes linked groups 2 and 3 from Zhong et al.(2013), which likely emerged as an artifact of sample absence from the middle United States and Central America. Two haplotypes from CR (H17 and H37) were shared with other countries. H17 was mainly found in Taiwan and H37 was in New Jersey and Texas. The two most common haplotypes, H67 and H68, collected from CR were directly connected to H17 and H37, respectively. These results suggest the Costa Rican *Ae. albopictus* population to be closely related with populations from Taiwan and eastern United States. In contrast, H72, a new haplotype found in our samples from the Atlantic basin of Panamá (Arco Iris, Provincia de Colón), was linked with H03, the most common haplotype reported for long COI sequences that has been found in China, Japan, Taiwan, Italy, and the west coast of United States (Zhong et al. 2013). Nevertheless, H03 was not present in our samples from Panamá and CR. The lack of connection between the two Panamanian haplotypes *Ae. albopictus* suggests that introgression of this mosquito into Panamá occurred two times (Clement et al. 2000), H72, being a haplotype whose spread might be limited to the Atlantic basin of the country. In this sense, our results partially support the expectation of more likely introductions in Panamá, as expected under a higher propagule pressure (Lounibos 2002). Nevertheless, although more than 10,000 houses were sampled in Panamá, *Ae. albopictus* was present in only 18, of which only 16 samples were analyzable, thus rendering impossible the more statistically powerful comparisons about diversity that would have benefited from a larger sample size from Panamá.

Ae. albopictus populations in Panamá and CR are likely not expanding, and in a genetic equilibrium, as indicated by a Tajima's D not being different from 0 (Tajima 1989). The K_{st} analysis showed that all populations were differentiated, even samples from CR and Panamá.

A limitation of our study was the heterogeneity in the mosquito sampling protocol, which ultimately reflects different procedures for entomological surveillance by the Costa Rican and Panamanian Ministries of Health. Nevertheless, this limitation came at the expense of cooperation for a better understanding of the phylogeography of a medically and economically important invasive mosquito vector species, *Ae. albopictus*. Although detected in both CR and Panamá, *Ae. albopictus* has not been directly implicated in dengue transmission in any of the two countries (Morice Trejos et al. 2010, Espino et al. 2011, Marín Rodríguez et al. 2013). Nevertheless, a study from Panamá City found up to 47% of *Ae. albopictus* pools positive to serotype 2 dengue virus, using molecular markers for flavivirus and RT-PCR (Espino et al. 2011), highlighting the potential for this mosquito species to become a major dengue virus vector in Central America.

The new vector control strategies targeting both *Ae. albopictus* and *Aedes aegypti* (L.) might increase the

costs for epidemic containment (Vazquez-Prokopec et al. 2010) and require a better understanding of *Ae. albopictus* ecology in the neotropics, mainly to improve entomological surveillance and control practices exclusively designed for *Ae. aegypti* (Morice Trejos et al. 2010, San Martín et al. 2010, Díaz 2012). Also, preliminary studies on *Ae. albopictus* larval ecology have shown its co-occurrence with many mosquito species unique to the Neotropics, e.g., *Limatus durhami* Theobald, *Haemagogus regalis* Dyar & Knab, *Trichoprosopon compressum* Lutz among other species with a wider distribution (Marín et al. 2009, Calderón Arguedas et al. 2012), and inquiring about the potential of these species to interact or even regulate the expansion of *Ae. albopictus* is a clear research priority. This is especially

necessary in light of the need to control *Ae. albopictus* in a pesticide-free manner, given the added value of organically grown products (Perfecto and Vandermeer 2008), like the pineapples of the farm where Costa Rican samples were collected. Similarly, modeling *Ae. albopictus* population dynamics in tropical environments is necessary to untangle any role that climate change may be playing in the expansion of *Ae. albopictus* and its interaction with *Ae. aegypti* (Tsuda and Takagi 2001, Chaves et al. 2012, Chaves et al. 2014).

Finally, our study highlights the need to further strengthen the regional cooperation in Central America to monitor the potential impacts of *Ae. albopictus* in the changing epidemiological patterns of dengue transmission, and to also formulate new control methods

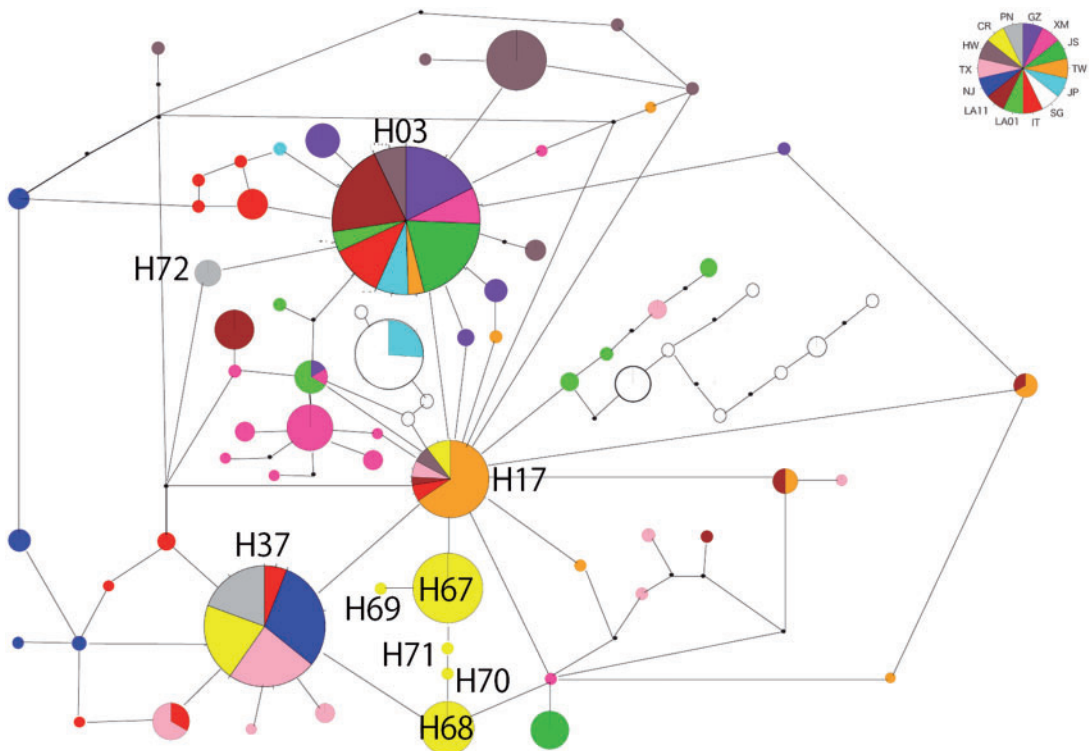


Fig. 2. Haplotype network based on mitochondrial COI (1,390 bp) of *Ae. albopictus* with all individuals collected in Costa Rica, Panamá, and the 12 populations reported by Zhong et al. (2013). Small black dots indicate hypothetical haplotypes not observed across the samples. Circle size is proportional to haplotype frequency, and lines between haplotypes indicate a mutational change. For haplotype codes, indicated by an H followed by two numbers, and population codes, indicated with colors in the figure, please refer to the text and Table 2, respectively. Further details about haplotype codes are presented in the Supp Table 3 (online only).

Table 3. Pairwise K_{st} estimates for the long (1,390 bp) COI gene sequences of *Ae. albopictus*

Pop	GZ	XM	JS	TW	JP	SG	IT	LA01	LA11	NJ	TX	HW	CR	PN
CR	1.18*	1.69*	1.32*	1.22*	1.37*	2.38*	1.61*	1.74*	1.56*	1.26*	1.61*	1.41*	–	–
PN	0.83*	1.78*	1.06*	0.85*	1.06*	2.99*	1.62*	1.92*	1.53*	0.96*	1.61*	1.24*	1.33*	–

Pop indicates the population, with rows indicating the focal populations Costa Rica (CR) and Panamá (PN), and columns indicating the background populations. Codes for background populations are presented in Table 2.

*Significant value ($P < 0.05$).

aimed at tackling the challenges that arise from the co-occurrence of *Ae. albopictus* and *Ae. aegypti* in a dengue endemic multinational region.

Acknowledgments

We are grateful to Prof. Marco Herrero (PIET, UNA, CR) for his role in promoting the collaboration behind this study. Dr. Brian Sedio (Smithsonian Tropical Research Institute, Panamá), Prof. Yukiko Higa (NEKKEN, Japan), and anonymous reviewers provided valuable comments on the analysis methods and discussion. Junko Sakemoto (NEKKEN, Japan) provided valuable administrative support. This study was funded by the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID), Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), Japan, and by Nagasaki University (Program for Nurturing Global Leaders in Tropical and Emerging Communicable Diseases).

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Received 6 February 2014; accepted 22 October 2014.